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13. ABSTRACT *(Maximum 200 Words)*

Parkinson's disease (PD) is caused by deterioration of the dopamine (DA) nigrostriatal system. Loss of DA can be induced experimentally by neurotoxic lesion of DA neurotransmitter producing neurons in the substantia nigra, or through lesioning DA axon terminals in the striatum with subsequent degeneration of their cell bodies in the substantia nigra. We have produced and characterized a new animal model of PD. Experimental PD has been induced by small unilateral, intrastriatal infusions of the neurotoxin 6-hydroxydopamine to produce partial loss of striatal DA. The striata of neurotoxin treated animals were examined morphologically and compared to the unlesioned, contralateral side at two time points following the treatment. Presynaptic striatal DA terminal losses were determined by evaluation of immunohistochemical expression of tyrosine hydroxylase. Differential changes in postsynaptic striatal DA receptor expression occurred at 1 and 4 weeks after neurotoxin exposure. Subsequent experiments examined the effect of exposure to a stressor on further exacerbation of DA receptor changes following the neurotoxin lesion. Neurochemical analysis of residual striatal DA following neurotoxin infusion and stressor exposure was performed in parallel, using HPLC of DA and its metabolites. Some tissue samples are stored and awaiting analysis to be performed over the next few months.

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Response to Reviewer's Comments:

Introduction:

A number of issues were raised by the review of our progress report for the 02 year of the grant. We have amended our presentation of the data and experimental descriptions as requested by the Technical Editor, based upon the reviewer's comments. A vertical line in the right-hand margin of this account designates those portions of the progress report that have been changed.

The reviewer raised 5 major areas of concern. These were: 1) the appropriateness of the partial unilateral lesion to the experimental design, and our failure to replicate established findings using this model, 2) a lack of explanation of the number of observations upon which the reported findings were based and methodological details, 3) the relationship between receptor binding measurements and immunohistochemical staining of dopamine (DA) receptor subunit proteins, 4) appropriateness of using the species-relevant TMT as a secondary stressor paradigm, and 5) productivity in the 02 year. We have addressed the reviewer's concern regarding the animal model in the next paragraph. The issues regarding the methodological details, ligand binding, and descriptions of technique are detailed within the Body of the report. Concerns about the level of productivity and use of TMT are presented in the Conclusions section.

Appropriateness of the animal model

Since the DoD approved the modification in our experimental design and our SOW (4/5/01), this objection by the reviewer is not relevant. *Our intent was to establish a preclinical animal model of Parkinson's disease (PD) that would enable examination of progressive nigrostriatal DA degeneration so we could study mechanism(s) of functional and structural recovery after subtotal damage of the nigrostriatal system.* Once a standardized model is established in our laboratory, we then could investigate pre- and postsynaptic determinants of DA nigrostriatal function. We would provide a second episode of stress to our preclinical PD model in an attempt to distinguish which portions of the nigrostriatal system under evaluation were the most vulnerable to subsequent damage. This objective has not changed.

There are numerous reports using partial 6-OHDA intrastriatal lesioned models from literature predating the 1989 report of cloning the D2 DA receptor, followed by identifying the other four DA receptor subtypes (as examples, Dunnett et al, 1981; Hefti et al, 1985; Snyder et al, 1985; Onn et al, 1986). A more recent group of publications assess the success of embryonic transplantation paradigms or interventions using growth factors in partial DA-lesioned PD models using 6-OHDA delivery to the striatum, substantia nigra, or median forebrain bundle (Rosenblad et al, 1999; Barneoud et al, 2000; Aoi et al, 2001; Bergstrom et al, 2001) and have contributed to our understanding of the plasticity of the nigrostriatal system. The seminal work of Anders Bjorklund's laboratory has clearly delineated functional aspects of the partially DA deafferented hemi-PD animal model, and reproducible end-point measurements in these studies examined tyrosine hydroxylase (TH) staining and cell counts in the substantia nigra, DA content of the striatum, skilled paw usage, and drug-induced rotation (Lee et al, 1996; Rosenblad et al, 2000; Kirik et al, 2001) when DA was reduced >60% in the striatum. These investigators found it was not possible to reliably assess behavioral changes when the animals were preclinical, which correlated to striatal DA levels less than 60% of control. Thus, our paradigm to produce a model of early stage PD is not replete in the literature. Furthermore, there are no studies examining changes in the five DA receptor subtypes after

partial DA depletion. While L-dopa treatment for PD is the clinical therapy of choice it induces incapacitating side effects, and thus recent additions to the drug arsenal target DA receptors directly. One such compound is pramipexal, a D3-preferring agonist (Piercey et al, 1996) that causes fewer dyskinetic side effects. Consequently our goal to examine changes in the expression and qualitative distribution of the different striatal DA receptor subtypes is timely. The only way to establish whether DA levels more tightly control a specific receptor subtype in the striatal milieu is to use tools that distinguish the different DA receptor proteins. We are uniquely positioned to perform these experiments because we have developed selective reagents that distinguish each of the cloned DA receptor subtypes (McVittie et al, 1991; Ariano and Sibley, 1994; Ariano et al, 1997a; b). This information is not in the published literature and consequently we are providing new data

We are characterizing a rat model of mild, "preclinical" PD, using unilateral infusions of two different neurotoxins, delivered into two different sites of the dopaminergic (DA) nigrostriatal system. Our experimental criterion is to establish the appropriate concentration of intrastriatal 6-OHDA and intranigral malonate that would yield a **50% loss of striatal DA levels** at 4 weeks following the neurotoxin infusions.

Body:

Preamble: We changed the neurotoxin protocol to establish a reproducible PD model, as explained in the amended SOW (letter dated 11/28/00) and approved via email by Dr. Karl Friedl (dated 4/5/01). This change was based upon the severe deficits that were induced in the rats following bilateral nigrostriatal lesions, even though the treatment was reduced in scope from published accounts (>80%) to 50% DA loss, and required substantial additional husbandry necessary to ensure that the animals thrived. Thus, this second year of the research plan initiated experiments using partial, ***uni***lateral 6-hydroxydopamine (6-OHDA) striatal lesions, commencing in December 2000. We enlisted the assistance of Dr. Timothy J. Collier (Rush-St. Luke's Presbyterian Medical Center) and Mr. Brian Daley (Rush) in surgically preparing and lesioning rats with the neurotoxin to expedite our scientific plan and accomplish as much of the SOW as possible in the remaining 02 year funding period. Dr. Collier and Mr. Daley were paid on a per surgical preparation basis for three sessions, providing consistency and capability to expose sufficient numbers of animals to the neurotoxin. We have successfully lesioned ~130 rats since that time using this arrangement, and the results from these rats provide the basis of the data reported here.

Table 1. Animal group census for the 02 year

Experiment	*Date*	*Survival*	*Quantity*	*Procedure*
Bilateral 6-OHDA	9/11/00	1 week	8	HPLC (CMS)[1]
Bilateral sham	9/21/00	1 week	3	HPLC (CMS)
Bilateral 6-OHDA	10/11/00	1 week	8	HPLC (CMS)
Unilateral 6-OHDA	12/5/00	1 week	4	IHF[2]
Unilateral 6-OHDA	12/13/00	1 week	2	IHF
Unilateral 6-OHDA	12/20/00	1 week	4	IHF
Unilateral 6-OHDA	1/10/01	1 week	9	HPLC (Yale)[3]
Unilateral 6-OHDA	1/10/01	1 week	7	IHF
Unilateral 6-OHDA	1/10/01	4 weeks	9	HPLC (Yale)
Unilateral 6-OHDA	1/10/01	4 weeks	9	IHF
Unilateral 6-OHDA + TMT	3/12/01	4 weeks + 1 day	6	HPLC (Yale)
Unilateral 6-OHDA	3/12/01	4 weeks + 1 day	4	HPLC (Yale)

Unilateral 6-OHDA + TMT	3/12/01	4 weeks + 5 weeks	9	HPLC (Yale)
Unilateral 6-OHDA	3/12/01	4 weeks + 5 weeks	4	HPLC (Yale)
Unilateral 6-OHDA + TMT	3/12/01	4 weeks + 5 weeks	7	IHF
Unilateral 6-OHDA	3/12/01	4 weeks + 5 weeks	4	IHF
Unilateral 6-OHDA + TMT	5/16/01	4 weeks + 5 weeks	7	HPLC (Yale)
Unilateral 6-OHDA	5/16/01	4 weeks + 5 weeks	5	HPLC (Yale)
Unilateral 6-OHDA + TMT	5/16/01	4 weeks + 5 weeks	8	IHF
Unilateral 6-OHDA	5/16/01	4 weeks + 5 weeks	4	IHF
Unilateral 6-OHDA	5/16/01	4 weeks	8	IHF

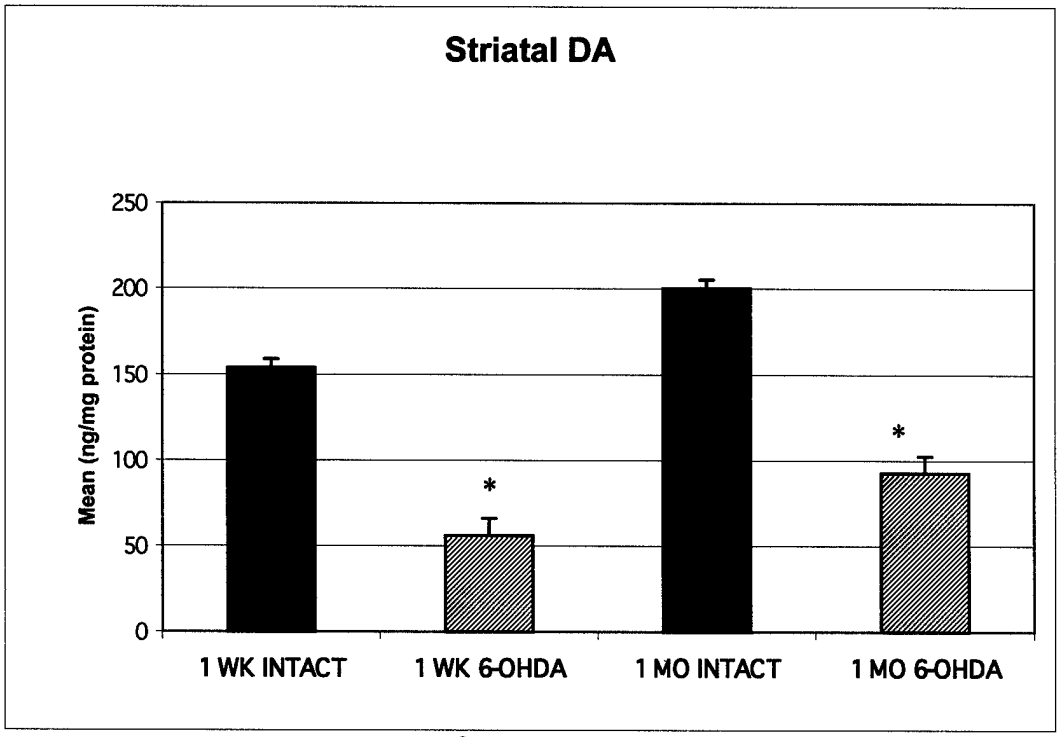
[1]HPLC was performed at the Chicago Medical School (CMS) with the assistance of Dr. Marina Wolf.
[2]Morphological assessment was performed using immunofluorescence (IHF) on striatal tissues.
[3]Dr. John Ellsworth (Yale) performed this analysis when the HPLC at CMS proved inadequate to meet our needs.

Pilot studies in the initial 1 ½ years of this proposal established that 6 μgm of 6-OHDA delivered stereotaxically into one site in the striatum produced the desired criterion of 50% depletion of striatal DA. The 6-OHDA (Sigma) was injected as a 2 μl bolus via a 26 gauge Hamilton syringe. The 6-OHDA was dissolved in ascorbate (5mg/25ml in sterile saline) to retard oxidation of the neurotoxin and was infused over a 2 minute time period to minimize structural damage due to the added fluid volume within the nucleus. The needle was left in place for an additional 2 minutes to reduce "wicking" of the neurotoxin up the track and then the syringe was retracted from the striatum. The 50% loss of striatal DA was established using HPLC measurements of tissue DA and DOPAC at 1-week and 4 week time points. The [DOPAC]:[DA] ratio provided an index of DA turnover and the status of the DA nigrostriatal system, as reported previously in the literature (Onn et al, 1985).

Task 1: *Commence unilateral striatal 6-OHDA infusions into young adult (175-200 gm), male rats using various infusion doses of the neurotoxin to establish the appropriate concentration to achieve 50% loss of striatal DA as detected using HPLC at the one-week time point after the surgery.* We performed a dose-response curve in the initial year and determined that 6 μgm (3 mg/ml in 2 μl) produced 50% loss of striatal DA. HPLC was performed at 1-week and 4-weeks. ANOVA and Fisher posthoc least squared difference were used to evaluate the data statistically. Significant differences (asterisks) were found in DA levels of the 6-OHDA treated striatum ($p<0.0001$) compared to the control side at both time points. (N = 9)

Figure 1.

A.



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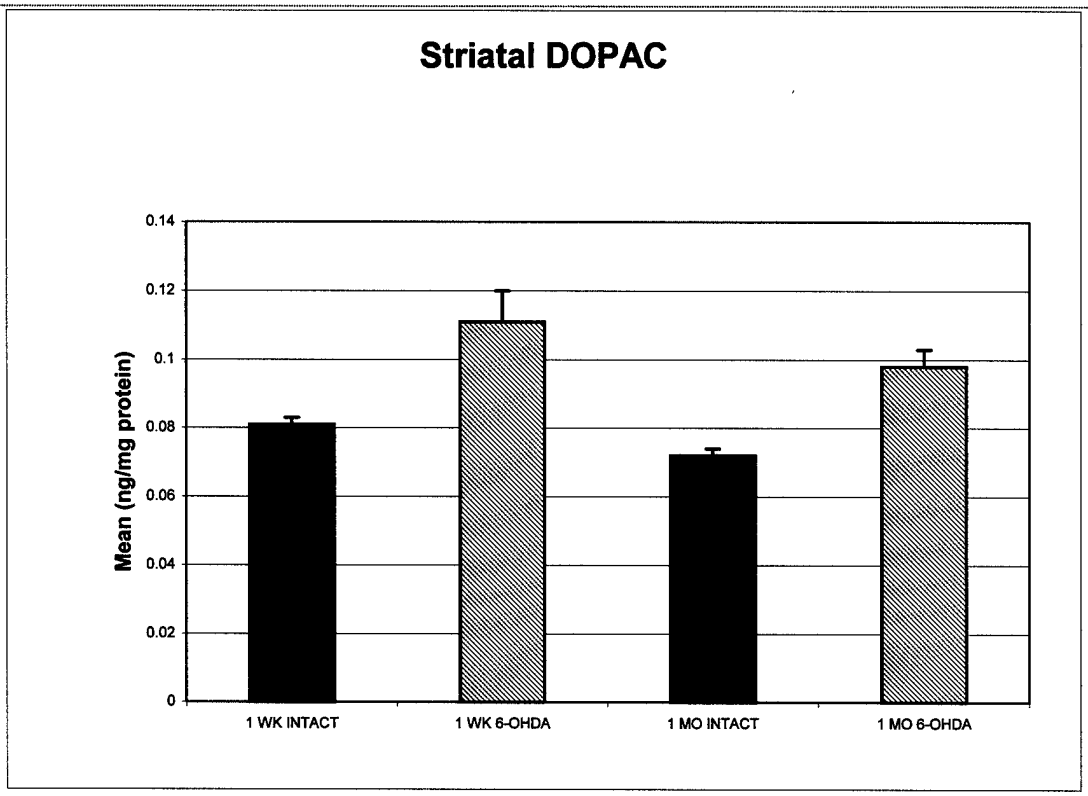
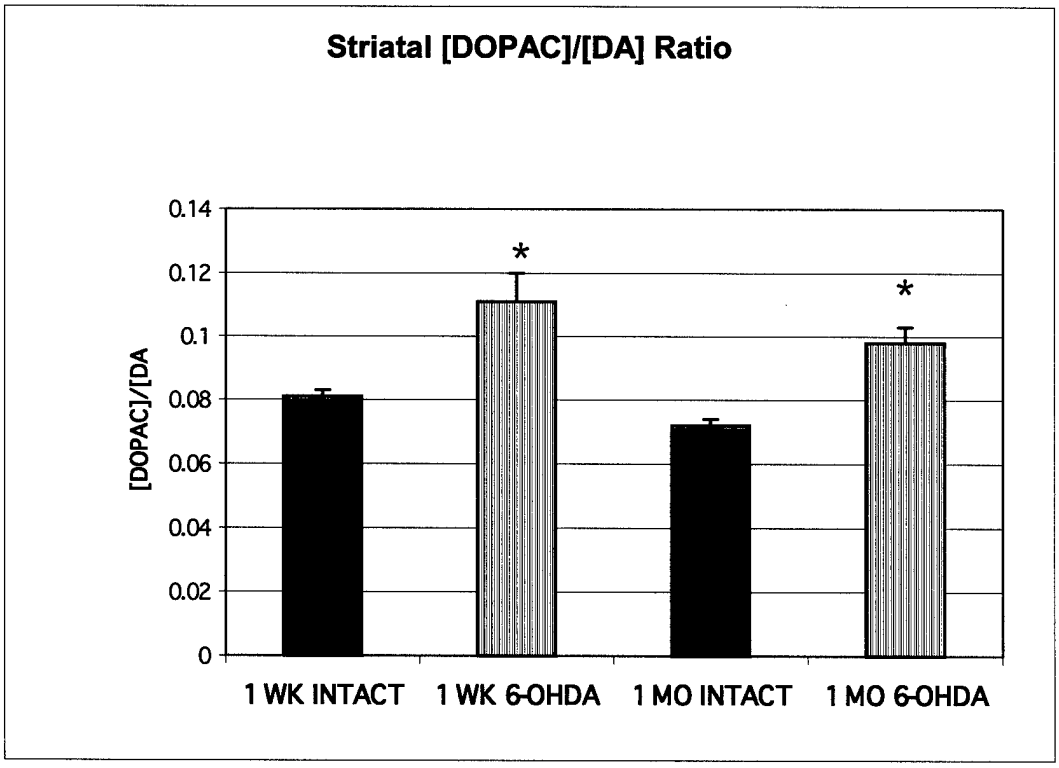


Striatal DA turnover was elevated significantly (asterisks) at 1 week ($p < 0.004$) and 4 weeks ($p < 0.03$). This is detected as the ratio of metabolite to neurotransmitter (e.g, [DOPAC] : [DA]).

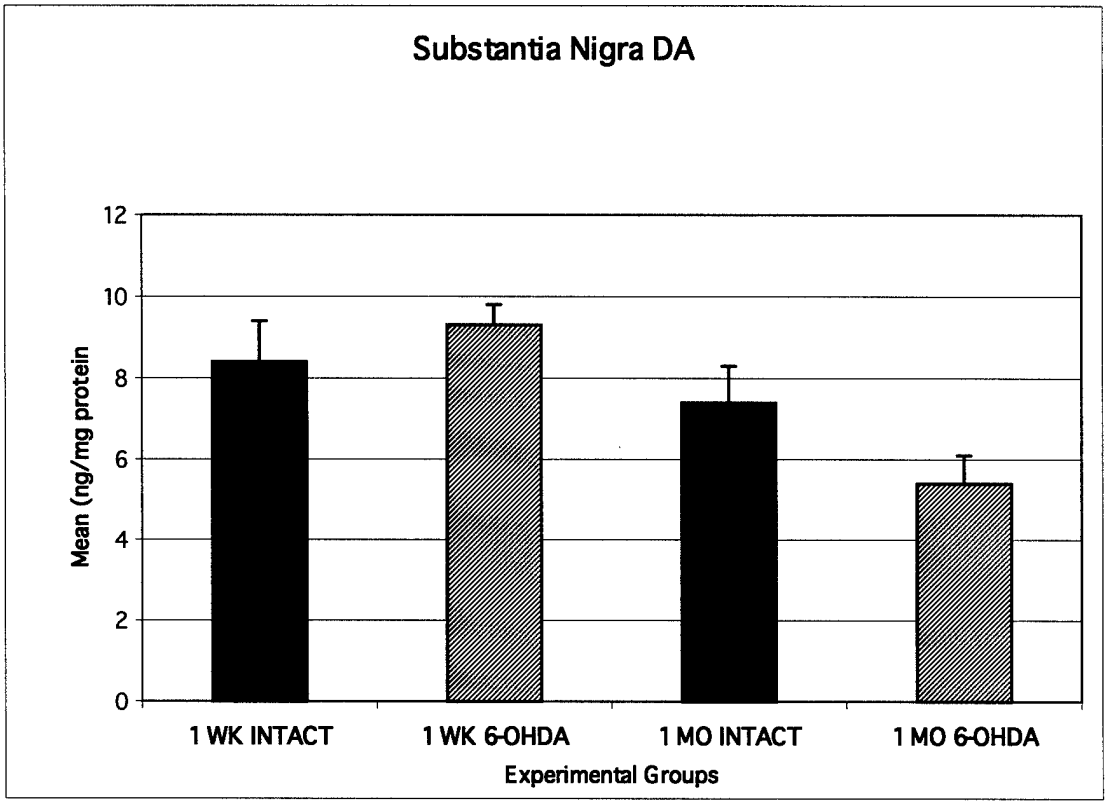
C.



We also evaluated the levels of DA, DOPAC and their ratio in the substantia nigra from these same animals. The loss within the midbrain following intrastriatal 6-OHDA infusions was blunted by comparison to that detected within the striatum. There were no significant differences. N = 9 for each experimental group.

Figure 2.

A.



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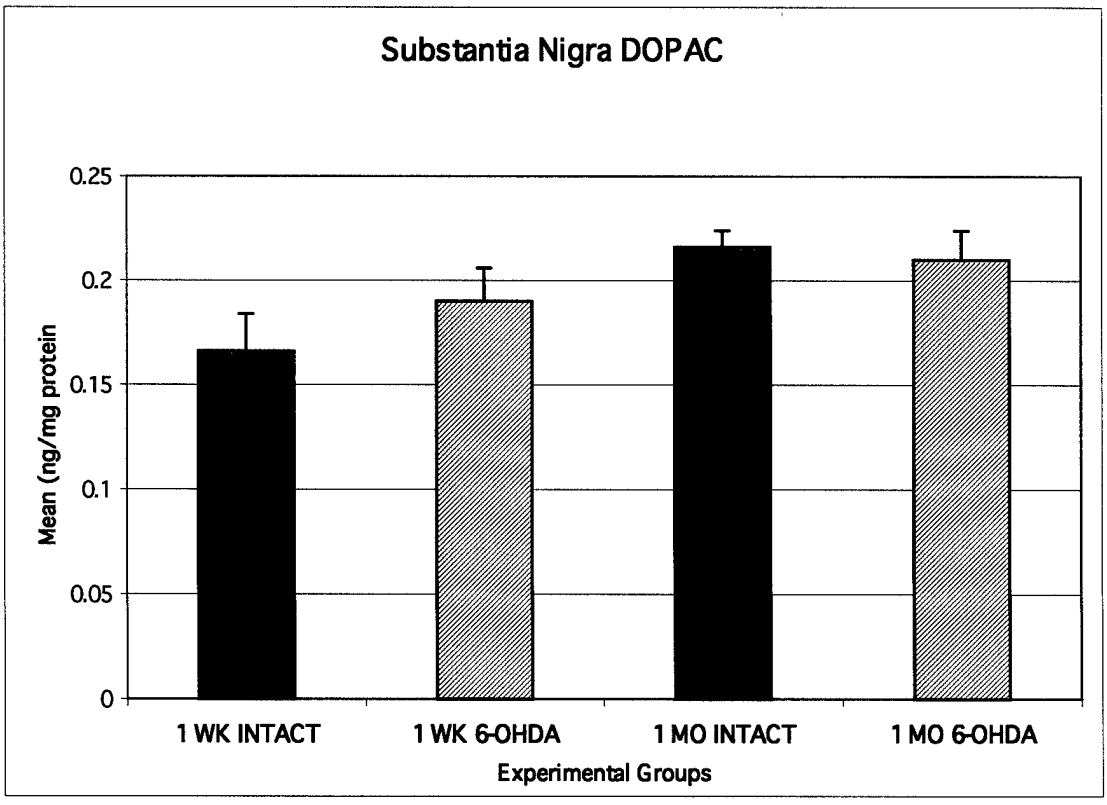
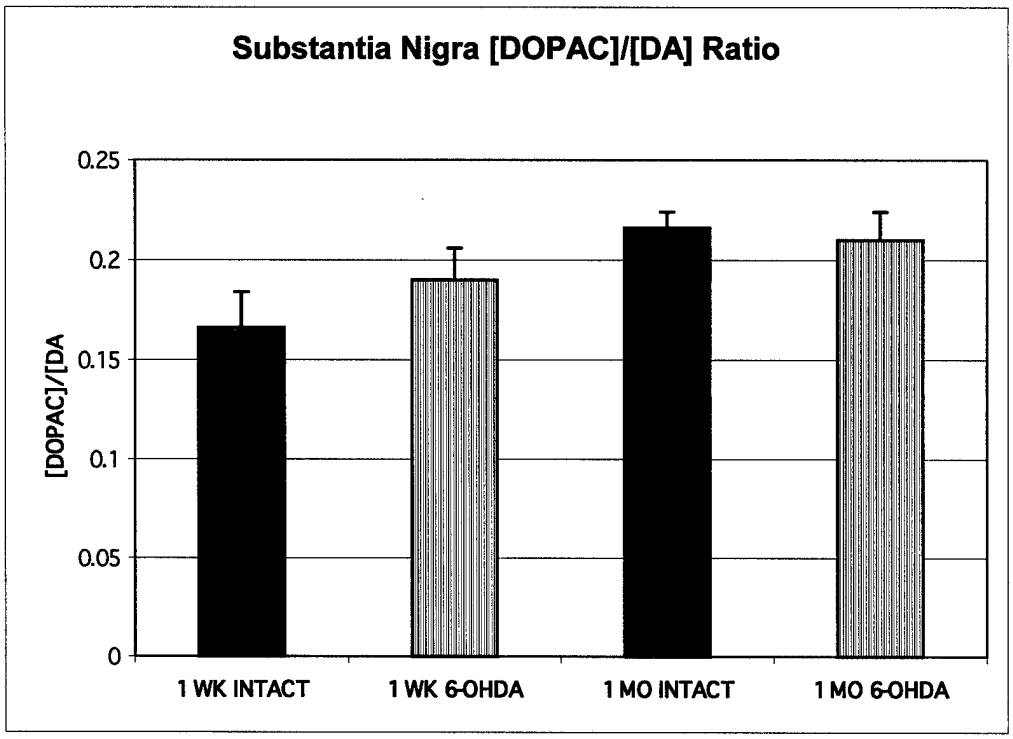


C.



The ratio of metabolite to neurotransmitter, ([DOPAC]:[DA]) was not changed significantly by the intrastriatal infusion of 6 μgm 6-OHDA at either time point. N = 9 for each group.

A caveat associated with performing unilateral, partial 6-OHDA striatal lesions is that the model does not cause a robust behavioral phenotype (Barneoud et al, 1995; Kirik et al, 1998). However, in the 2-3 initial hours following the neurotoxin infusion, animals will turn in tight circles without provocation or drug administration, and curl into "balls" (nose to tail posture) toward the side of the infusion due to the imbalance in striatal DA. This was noted for the neurotoxin-lesioned animals. But behavioral parameters could not be assessed reliably (much like the preclinical PD patient) due to the subtlety of our neurotoxin-exposure paradigm. Conversations and discussions held at the DoD Workshop in Potomoc, MD in late March supported and confirmed our suspicions that rats with partial, unilateral DA losses lack a measurable behavioral phenotype. We therefore decided to forego behavioral evaluations, and at the same time we decided to complete the entire series of 6-OHDA studies before changing to our second neurotoxin regimen, malonate. Malonate infusions will be directed into the substantia nigra to yield a 50% loss of striatal DA. These experiments will be performed in the third year of the research program and will follow an analogous design sequence as described for the 6-OHDA studies.

Task 2: *Evaluate the morphological staining for the five DA receptor subtypes and the cyclic AMP second messenger within the neurotoxin-lesioned and intact striata, 1 week following the 6-OHDA-infusion that yielded a 50% striatal DA loss as determined in Task 1.* Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of catecholamines. It is a standard procedure to visualize TH staining within DA-containing regions of the brain to assess terminals (in the striatum) or cell bodies (in the substantia nigra). We used TH immunofluorescence to evaluate the loss of striatal DA in rats, one week following intrastriatal infusion of 6-OHDA.

Sections were examined using epifluorescence microscopy. Digitized images for control or lesioned striatal areas were matched to similar regions in the dorsal half of the nucleus. Image acquisition parameters for each antisera staining experiment were optimized using the control sections, then the identical settings were used to obtain images from the matched lesioned side thus, normalizing the exposures with respect to the control striatal sections. Image acquisition followed a specific sequence in the coronal tissue sections, from dorsolateral, dorsomedial, ventrolateral to ventromedial quadrants of the striatum, in each of the tissue sections mounted on the microscope slide. Data were stored without enhancement and analyzed off-line. This experimental protocol enabled comparisons to be made between the control and lesioned striata in each animal for each antisera evaluated. The fluorescent staining reactions in paired images from equivalent striatal regions in each control and respective lesioned striata were converted to histogram luminosity values using Adobe Photoshop™ to assign a numerical value to the gray level of staining intensity. The median values of the histogram for images obtained in a specific antisera experiment were expressed as the lesioned side luminosity value divided by the control side luminosity value, and reported as a percentage increase or decrease. Values reported are means ± s.e. and N's indicate the number of animals sampled. Statistical analyses (paired t-tests) were performed on the luminosity values when data were obtained from 3 or more different 6-OHDA-lesioned animals at either the 1-week or 4-week time points, stained for individual antisera. Differences were considered statistically significant when $p<0.05$.

Figure 3. TH immunofluorescence was evaluated using a monoclonal antisera (Chemicon, Temecula, CA) directed against the enzyme 1 week following 6-OHDA intrastriatal infusion. Frozen 10 μm thick coronal tissue sections were immersion-fixed in 4% paraformaldehyde for 10 min at room temperature, then processed for routine TH immunofluorescence. Fluorescence was detected throughout the substance of the intact striatum, however myelinated fiber bundles of the internal capsule that penetrate the nucleus are seen as black, unstained irregular circles. The lesioned striatum was examined subsequently, and images were acquired using the identical settings as the intact side. There is a considerable attenuation in the fluorescence signal in the 6-OHDA exposed striatum. The calibration bar is the same for both images.

TH staining for the 1-week time point is based upon morphological evaluation of four different animals, and 3 different "runs" of the immunofluorescence reaction per rat. The gray scale image was converted to a histogram function using Adobe Photoshop™, then plotted as a numerical representation of the change in DA terminal innervation of the neurotoxin-treated striatum with standard error bars. This is demonstrated below for the 1-week and 4 week lesion duration time points.

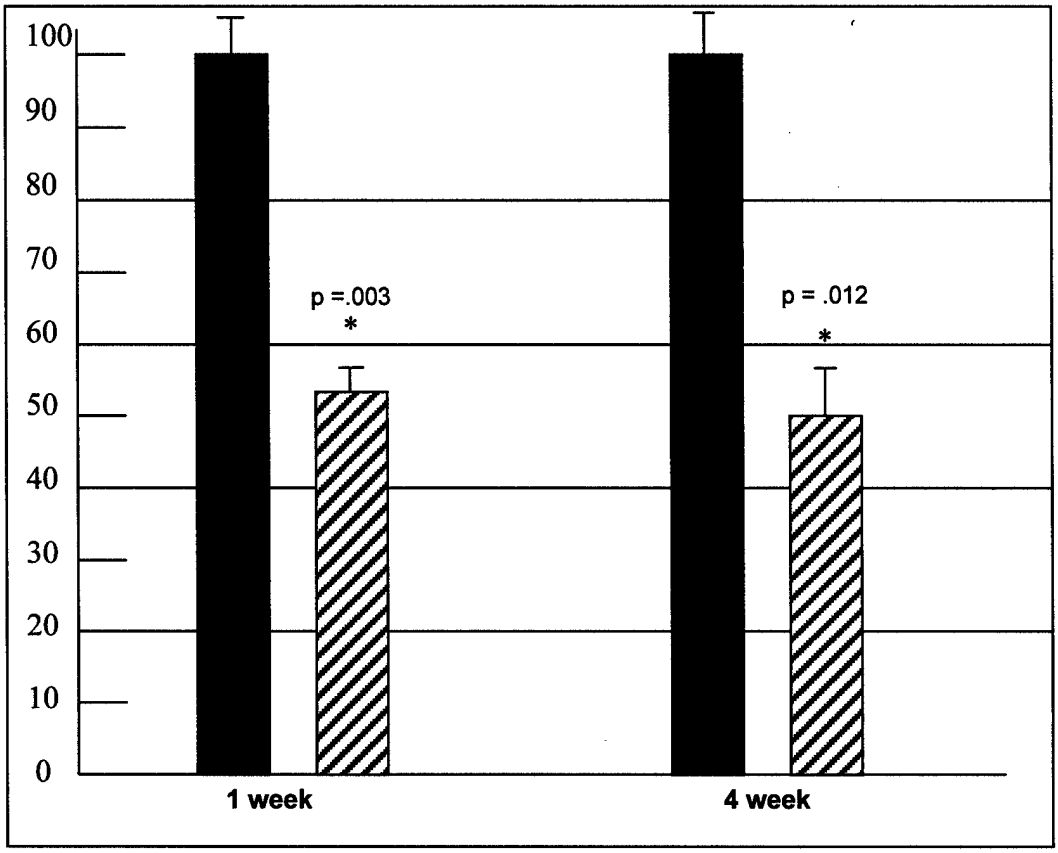
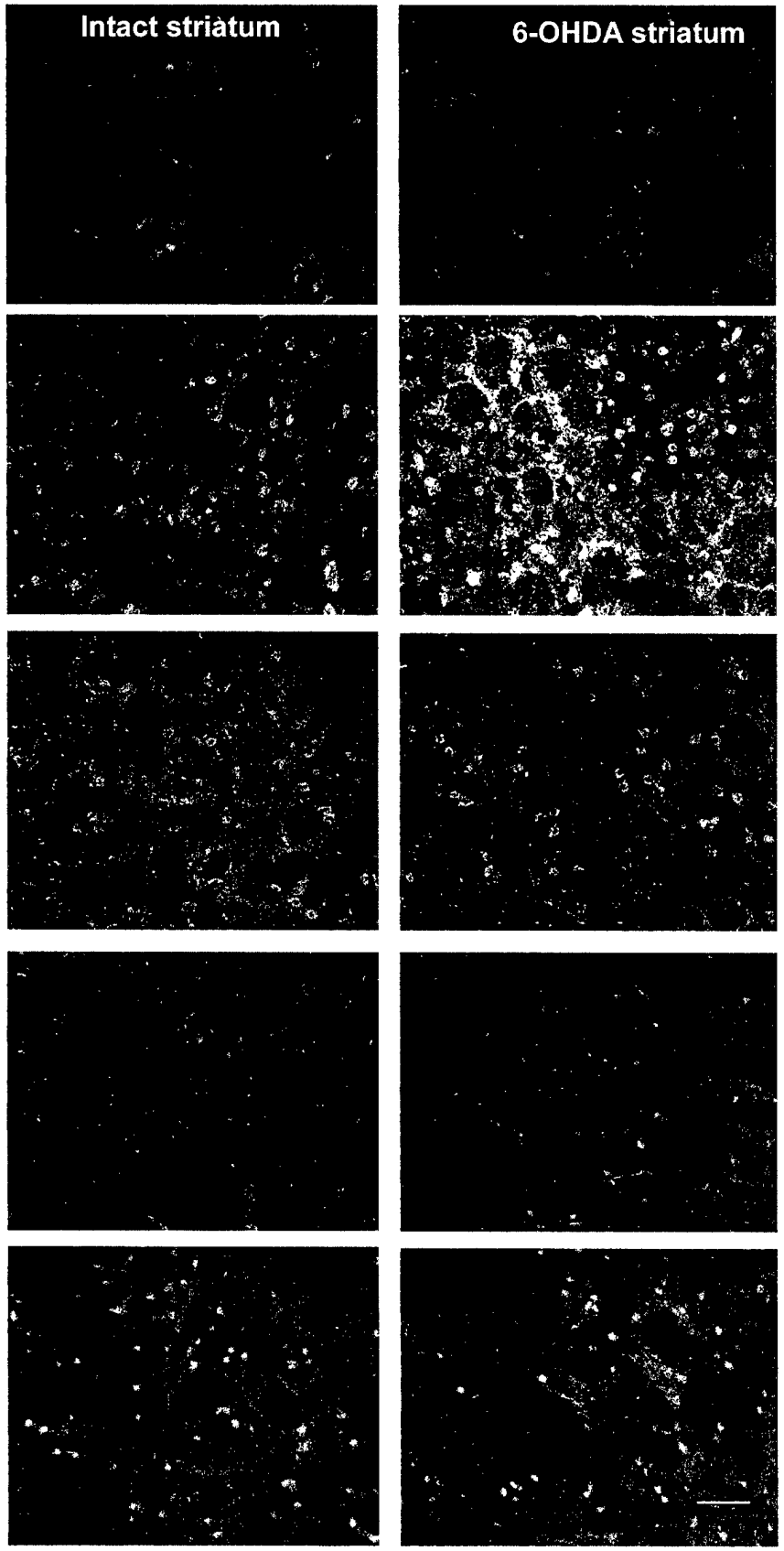


Figure 4. Luminosity values were determined using the histogram function in Adobe Photoshop™ from the median gray scale intensity derived from the fluorescent staining of TH in striatal tissue sections. Images were paired from control (solid colors) and 6-OHDA lesioned (patterned colors) regions within each mounted microscope section at 1 week (n = 4) or 4 weeks (n = 9) after the intrastriatal infusion. The TH luminosity value of the control was assigned as 100%, and the TH staining intensity of the lesioned side is presented as a percentage loss from the control side. In the 1-week lesion example, the TH fluorescence median intensity of the control is 166 $\pm$ 9.4 and was assigned as 100%. The lesioned side median luminosity value was 82.2 $\pm$ 7.1, corresponding to 54.7% decrease in TH fluorescence on the lesioned side. For the 4-week sample, the TH staining of the control was 179 $\pm$ 8.4 and was assigned as 100% in the histogram. The TH signal was 87.4 $\pm$ 6.3 on the lesioned side, which corresponds to 48.8% loss on the lesioned side.

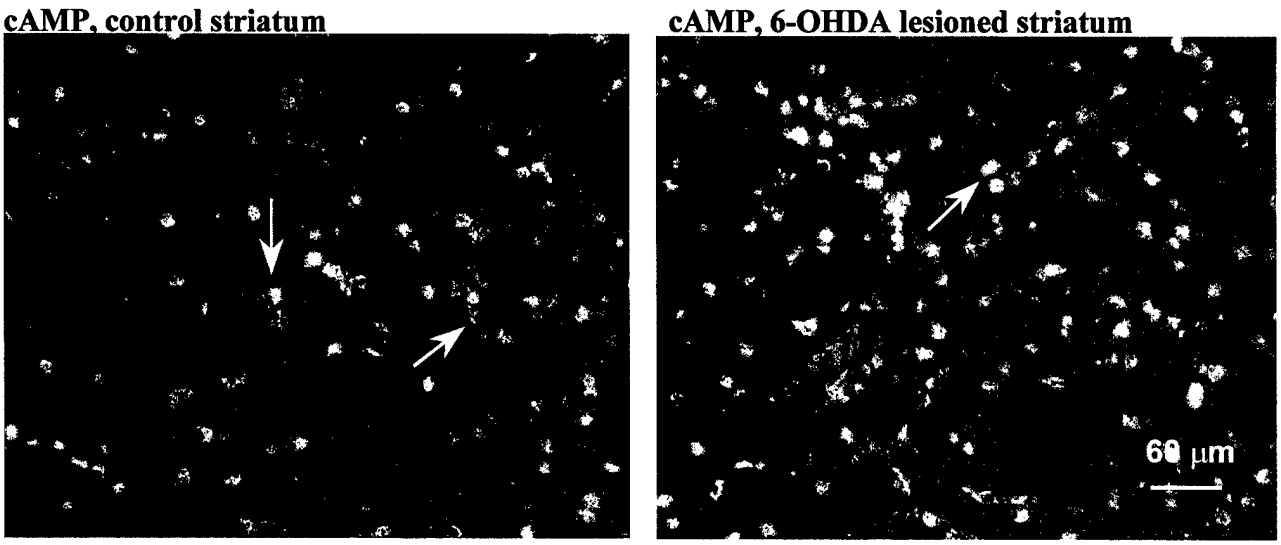
Figure 5. Immunofluorescence was used to study staining patterns for striatal DA receptor proteins, 1 week following intrastriatal infusion of 6 μgm of 6-OHDA. The control (DA intact) striatum is pictured to the left in each pair, while the neurotoxin-treated sample is on the right. Luminosity values are calculated as lesion signal/control signal. Calibration bar = 100 μm for all images.



The observations of DA receptor protein subtype staining are based upon four different 1-week lesioned animals. Immunofluorescence for each DA receptor subtype was performed a minimum of 2 times in each animal. Staining for the DA receptors was detected within the thin cytoplasmic rim of medium diameter striatal neurons (this population is mainly the projection cells), and the occasional large-sized interneuron. Processes also were reactive for each of the DA receptors, as the striatal neuropil exhibited fluorescent staining signal in comparison to the non-stained myelinated fiber bundles of the internal capsule that penetrate the parenchyma of this nucleus in the rat. Qualitatively, the D1 and D2 receptor subtypes are the most prevalently expressed within the striatum, and only the D2 and D3 receptor subtypes demonstrated consistent changes at this early time point; D2 protein staining was elevated, while the signal for the D3 subtype was decreased on the lesioned side in comparison to the control (DA intact) striatum. It is important to be very circumspect in the interpretation of changes in protein immunofluorescence. This is because the indirect immunofluorescent method, e.g., using secondary, fluorescently labeled antisera to observe the primary DA receptor reagent or cyclic AMP, cannot be precisely quantitated. A standard curve of fluorescence cannot be generated from this experimental paradigm. While commercial products guarantee a "high specific labeling" to an F'ab fragment, it is not possible to assess how many of these labeled secondary antisera actually bind to the Fc fragment of the primary antisera. Thus we only compare immunohistochemical experiments that have been performed concurrently, within a single run.

The loss of striatal DA will have profound, and complex effects on the postsynaptic expression of its different receptor subtypes (Minowa et al, 1994; Hirsch 2000, Hwang et al, 2001), and is dependent upon the size of the DA-depleting lesion, duration following the DA loss, how near to the toxin infusion site the morphological analysis is made, and the post-lesion drug treatment regimen. It should be noted that receptor binding studies, using DA receptor family ligands would not distinguish the individual protein subtypes, because the pharmacological tools that will select D1 versus D5 subtypes (D1 family), or D2 versus D3 and/or D4 (D2 family) are still not available. This has been a major stumbling block in assessment of which specific DA receptor is most vulnerable to loss of DA and should be preferentially targeted by drug treatment therapies in PD. As a consequence, radioligand binding does not provide the answer we are attempting to address, which is; *Which of the five striatal DA receptor subtypes are most vulnerable to subtotal DA lesions*? Another caveat that we must consider is that receptor protein staining may distinguish subtypes which are not functionally active but do present the appropriate epitope binding site for recognition by our primary antisera. This can be detected experimentally as a strong cellular signal for the DA receptors, due to the synthesis of the protein within the cytoplasm. The neural circuitry and subcellular organization of the striatum (Hanley and Bolam, 1997) does not show a plethora of axosomatic synapses; most connections occur distally following the second branching on the shafts and spines of dendrites. The cellular concentrations of the receptors drop off as they are dispersed away from the perikaryon retrogradely throughout the dendritic tree of the striatal neuron, or anterogradely transported to the terminal fields. These distribution patterns are not unique to the DA receptors, as analogous findings have been reported for other systems (van den Pol 1994; Daunt et al, 1997; Hall and Soderling, 1997; Martin-Negrier et al, 2000; Scannevin and Huganir, 2000; Smith et al, 2001). The final determination of functional impact of DA loss on DA receptor expression must be conservative and consider these caveats. The reviewer suggested we initiate studies to examine the relationship between DA receptor binding and immunohistochemical staining. This is not a trivial proposition. We feel this is well beyond the scope of this grant and we do not have the appropriate radioactive licensing to procure and dispose of radiation in the laboratory. However, if the DoD feels this is sufficiently critical to explore, we will submit a financial supplement to the present grant to initiate these investigations, as the cost of radiation disposal is significant.

Figure 6. The second messenger, cyclic AMP, was examined within the striatum, 1 week following intrastriatal neurotoxin exposure. Cyclic AMP provides an estimate of basal metabolic rate, as it is intimately involved in the production of ATP within cells. It also transduces D1/D5 effects. The data suggests that the density of stained cells (neurons [arrows] and glia), are increased following intrastriatal neurotoxin, and the intensity of staining likewise is elevated. The calibration bar is 60 μm and applies to both images. The median luminosity values for cyclic AMP at 1 week was 116.5% ± 7 (p=.001, n = 4) on the lesioned side compared to the DA intact striatum.



Summary of Striatal Immunofluorescence at the 1-week Evaluation Point

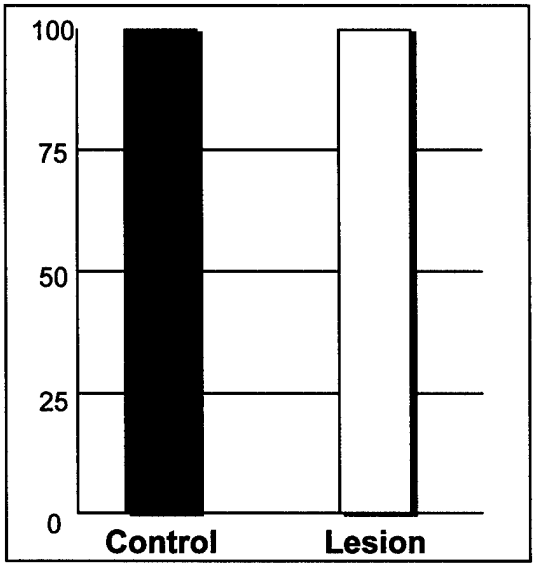
We routinely analyzed the striatal region rostral to the site of the needle track to avoid confounds due to tissue disruption by the infusion of the 2 μl of neurotoxin. Within an antisera stain, there was some variability, but overall the immunofluorescent outcome was consistent at this duration following the neurotoxin infusion. D2 and cyclic AMP were elevated on the lesioned side; D3 was decreased on the lesioned side in our studies. We are preparing to evaluate striatal samples using western analysis for basic quantitation of our findings. Both D2 and D3 receptors work through inhibition of cyclic AMP production and elevation of other transduction pathways (PIP, Ca^{+2}). We would anticipate that due to the more prevalent expression of the D2 receptor in the striatum, as opposed to the D3 receptor, that cyclic AMP levels should be diminished on the lesioned side. Our data clearly showed cyclic AMP staining is elevated consistently in the DA-depleted striatum. Thus, some other non-DA receptor mediated mechanism that is positively linked to adenylyl cyclase must be operating in the preclinical PD model.

Task 3: *Evaluate the morphological staining for the five DA receptor subtypes and the cyclic AMP second messenger within the neurotoxin-lesioned and control striata, 4 weeks following the neurotoxin infusion. HPLC evaluation of striatal and substantia nigra samples was performed on additional animals prepared in this surgery.* The analysis is based upon nine different animals, with staining for each DA receptor subtype and cyclic AMP performed a minimum of two times in each animal.

Figure 7. Luminosity histogram plots for the five DA receptor proteins and cyclic AMP are presented, 4 weeks after intrastriatal infusion of 6 μgm 6-OHDA. Values are plotted as the signal derived from the lesioned striata divided by the control intensity. The qualitative expression for the five subtypes was not changed from that displayed in figure 5 for the 1-week experimental time point, and therefore we have not reproduced images for this data.

D1 Subtype

102.3% ± 1.5

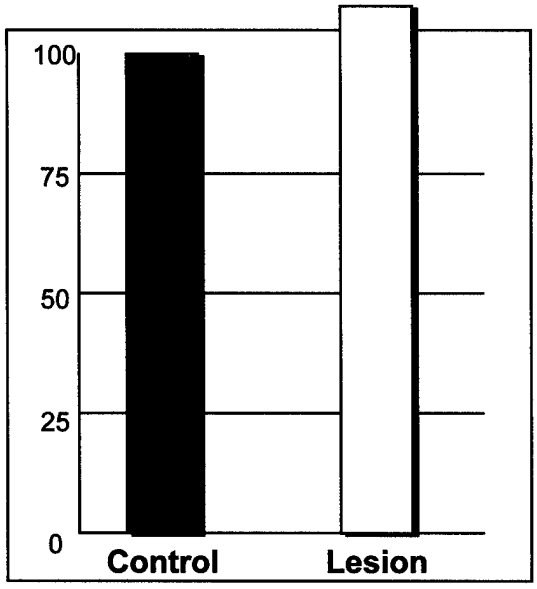


p = 0.48

N = 9

D2 Subtype

113.1% ± 3.1

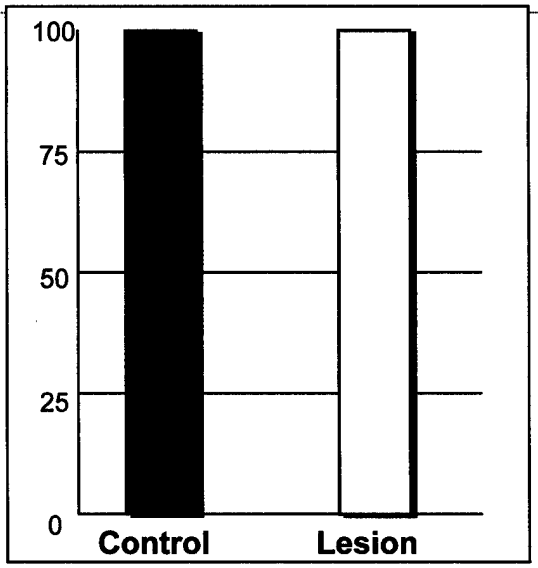


p = 0.08

N = 9

D3 Subtype

100.8% ± .8

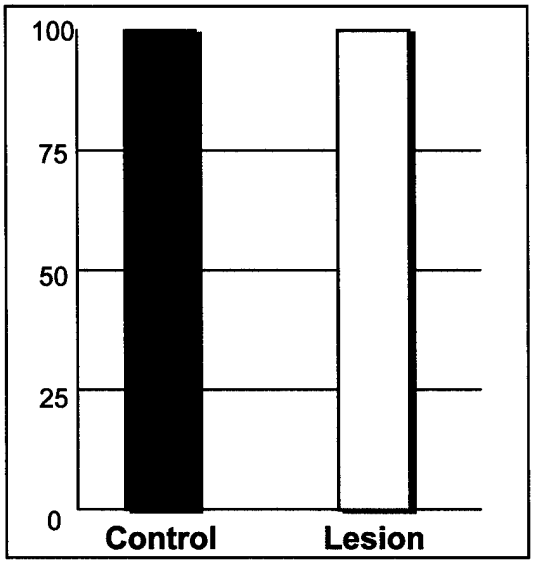


p = .95

N = 9

D4 Subtype

97% ± 1.5

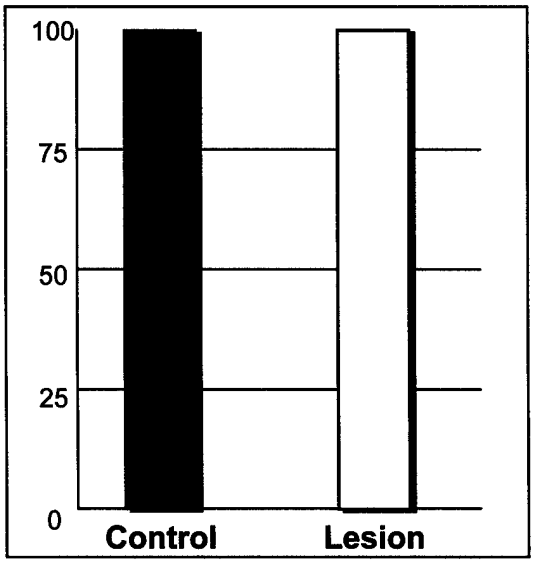


p = .78

N = 9

D5 Subtype

101.9% ± .81



p = .89

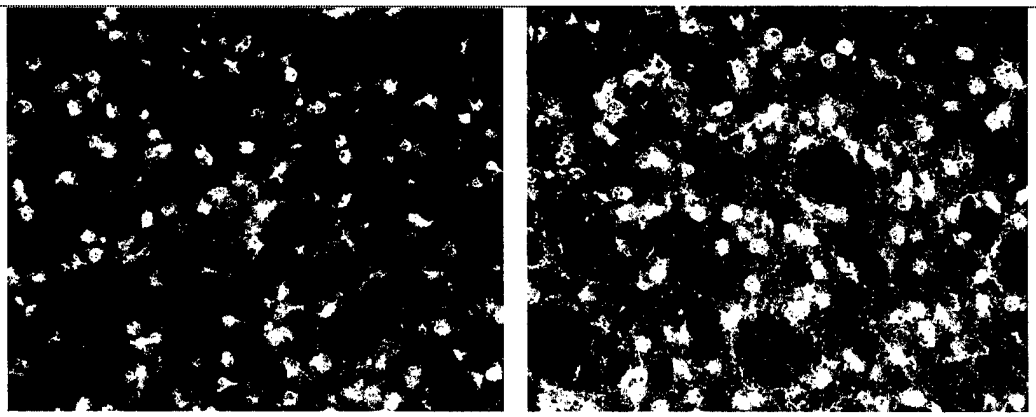
N = 9

Cyclic AMP

116.1% ± 4.8

p = 0.05

N = 9



Control **Lesion**

Summary of Immunofluorescence at the 4-week Evaluation Point

The DA receptor staining intensity and qualitative expression patterns have stabilized at this longer time point, with only the D2 subtype expression visibly changed by the deafferentation. This has been reported numerous times in the literature using large (>90%) DA-depleting lesions on D2 binding and mRNA levels (c.f. Gerfen et al, 1990 and references therein). Another consistent finding is that the cyclic AMP second messenger is elevated on the DA deafferented side, in comparison to the control striatum. This outcome reached statistical significance, and it suggests that basal metabolism has been altered by the DA loss, and/or that another adenylyl cyclase receptor-linked mechanism is up regulated. Since neither D1 nor D5 levels are enhanced (both are positively linked to cyclic AMP production), an alternative, non-DA receptor mechanism must be operating. We will corroborate these findings using western analysis.

Secondary Stressor Event

Based upon recent literature and enthusiastically supported in discussions at the DoD March 2001 workshop, we decided to employ a species-relevant stressor to elicit potential secondary neurodegeneration of the DA nigrostriatal pathway. Rats exposed to small quantities of TMT, the active compound in fox feces, exhibited vigorous repulsion to the odor, burrowing in their cage bedding, and group-huddling at the opposite end of the cage from the TMT-spotted filter paper (see procedural description below). This robust performance was detected in all rats, throughout the treatment, and was documented photographically. Since we do not have the capability to document the entire behavior using video cameras or activity cages, as suggested by the reviewer, we provided a digital camera image of the remarkable response by the naïve rats to the odorant (figure 8).

We are in the process of determining whether a subsequent exposure with a secondary stressful event following the partial unilateral DA lesion will provoke further changes in the nigrostriatal DA system. We have used the predator odorant, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) to provide a species-relevant aversive stimulus (Wallace & Rosen, 2000; Williams 1999). Recent work has demonstrated that TMT has unique effects on the DA systems of the brain (Morrow et al 2000a & 2000b) and its use requires no previous shaping of the experimental animals; the vigorous response to the odorant appears innate, even in lab rats. Previous rodent studies have demonstrated clearly that acute emergence of PD behavioral symptoms during stress could be produced following

extensive damage of the nigrostriatal DA system, concealed in the preclinical phase of the disease (Snyder et al, 1985). Thus, exposure to stressors may lead to significant changes in striatal DA receptor expression patterns, further behavioral abnormalities, and changes in DA and mitochondrial functional indices in the substantia nigra. Our model and experimental paradigm are much more selective in producing DA nigrostriatal degeneration, and are designed to approximate the sequelae that may be encountered by military personnel potentially exposed to neurotoxins in the combat theater or during military service (the initial toxic insult to the DA system), followed by a subsequent stressor due to other environmental or emotional life changes (the secondary insult) triggering onset of PD in vulnerable individuals. Our experiments will determine which elements of the nigrostriatal DA system are especially at risk at specified time points after low level neurotoxin exposure that may not produce overt behavioral phenotypes, providing a coherent description of susceptibility for PD and elucidate potential biomarkers for therapeutic interventions after a secondary stressor event.

Task 4: *Evaluate the effect of a second stressor, induced by exposure to the predator odor TMT, on the HPLC parameters at 4 weeks following the 6-OHDA infusion. Animals were killed 24 hours after one TMT treatment of 1-hour duration.* Animals exposed to TMT (Pherotech, Vancouver BC) were transferred to an isolation room in their home cages. TMT was applied undiluted (30 µl) onto Whatman #1 filter paper placed onto the top of the wire screen lids of the cages. The papers were left in place for 1 hour, then the filter paper rounds were collected into a plastic bag and sealed. The experimenter did not remain in the room, as this might confound the experience for the rats. Observations could be made through a viewing window in the door of the isolation room. Animals remained in the isolation room following the TMT exposure for the remainder of the day, and the following morning the bedding, cages, water bottles and food were replaced before the animals were returned to the home room in the animal facility. Animals typically exhibited increased movement when the cages were transferred into the new environment (circling, rearing, sniffing). They were interested in the filter paper when it was placed atop the cage. When the TMT solution was squirted into the center of the filter paper, the initial sniffing and rearing behavior was replaced rapidly with racing to the opposite end of the cage, burrowing beneath their litter, grooming the snout and vibrissae, and curling into "balls" with their cage-mates (Figure 8). This behavior lasted through the initial 10 minutes of the odorant presentation. Behavior gradually got less frenetic and was replaced by exploration of the filter paper circle, but continued return to the opposite end of the cage. The reaction to the TMT became less vigorous with repetition, and the last series of animals actually "attacked" the filter paper circle, bringing it inside the cage and "killing" the predator odor by shredding it. TMT-treated rats continue to exhibit hyperactivity during the stay in the isolation room, and seemed more nervous and "jumpy" during the entire duration of the trials.

We designed two different TMT experimental trials. These are depicted below for comparisons.

Acute Group	*6-OHDA*	*Week 1*	*Week 2*	*Week 3*	*Week 4*	*Week 5*
Control n = 4	3mg/ml; 2 ml	--------	--------	--------	*	
TMT n = 6	3mg/ml; 2 ml	--------	--------	--------	*	

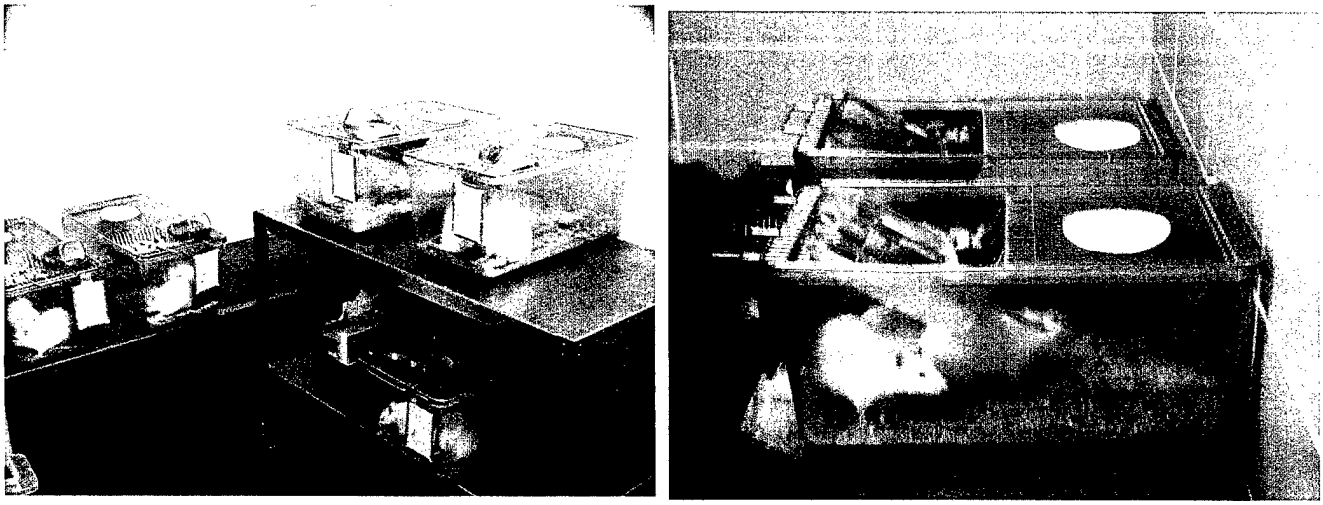
* TMT exposure, 30 µl for 1 hour

••••••Animals sacrificed, at 24 hours following TMT treatment

Chronic Group	6-OHDA	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Control n = 5	3mg/ml; 2 ml	--------	--------	--------	--------	--------	
TMT n = 9	3mg/ml; 2 ml	*	*	*	*	*	

*TMT exposure, 30 µl for 1 hour, returned to home room the following morning after changing bedding, cages, etc.
••••••Animals sacrificed, at 1 week following the last TMT treatment

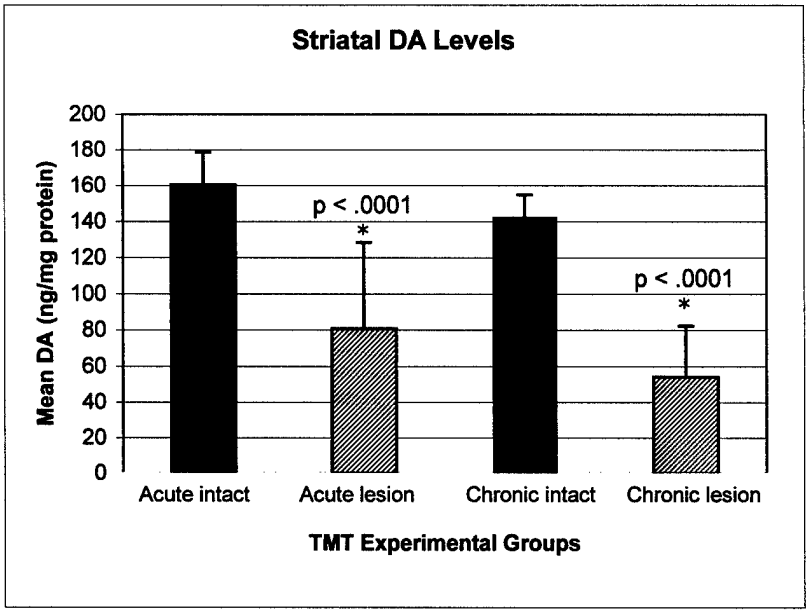
Figure 8. Neurotoxin-treated rats responded vigorously to the predator odor, TMT. Filter paper circles were placed on top of the wire cage lids and 30 µl of the TMT was spotted onto the paper. Note that animals have moved to the opposite end of the cage (left panel). A zoomed image of the rats in the right side panel shows enhanced "whisking" of the vibrissae associated with increased sniffing, and tentative exploratory movements toward the TMT spotted filter paper.

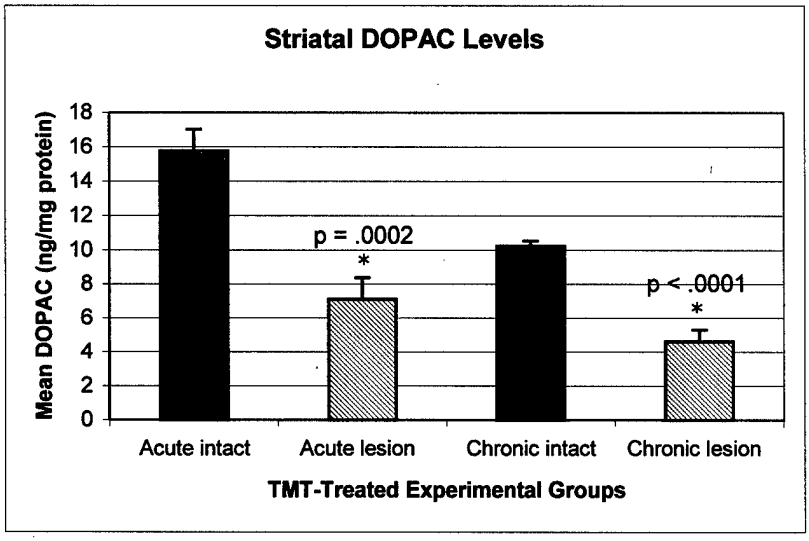


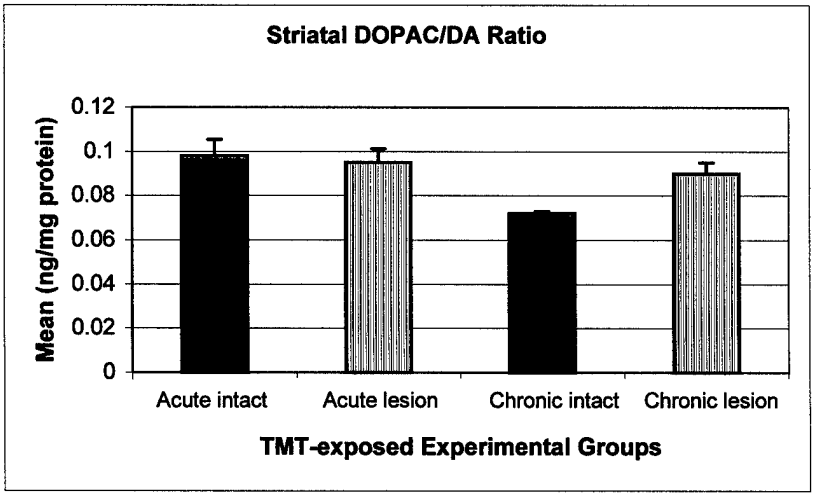
Task 5: *Evaluate the effect of the TMT stressor on the morphological and HPLC parameters at 4 weeks following the 6-OHDA lesion. Animals were killed after five 1-hour TMT treatments given at weekly intervals.*

We have performed HPLC determination on the rats exposed to an acute (e.g., one TMT treatment followed by sacrifice 24 hours after the exposure), or chronic (5 TMT treatments, delivered once/week, followed by sacrifice 24 hours after the last exposure). The striatal levels of DA, DOPAC and the ratio of [DOPAC] to [DA] are shown in figure 9 on the next page. The intact side is shown by the solid bar graph, while the lesioned side is patterned for the acute or chronic TMT exposure conditions. Statistical significance between acute experimental groups, or between the chronic experimental groups for DA levels, DOPAC levels, and the ratio of metabolite to neurotransmitter was compared by ANOVA and Fisher posthoc least squared difference. Asterisks denote significant differences, and the p value is included. Based on this initial evaluation, TMT appears to diminish the production of striatal DOPAC levels, but has little affect on DA turnover. N = 4 in each group. The experimental design will need to be explored in a larger group of animals, and we will also include a more frequent chronic exposure series (e.g., 2 times per week).

Figure 9.


Striatal DA Levels
Mean DA (ng/mg protein)
200
180
160
140
120
100
80
60
40
20
0
p < .0001
*
p < .0001
*
Acute intact
Acute lesion
Chronic intact
Chronic lesion
TMT Experimental Groups

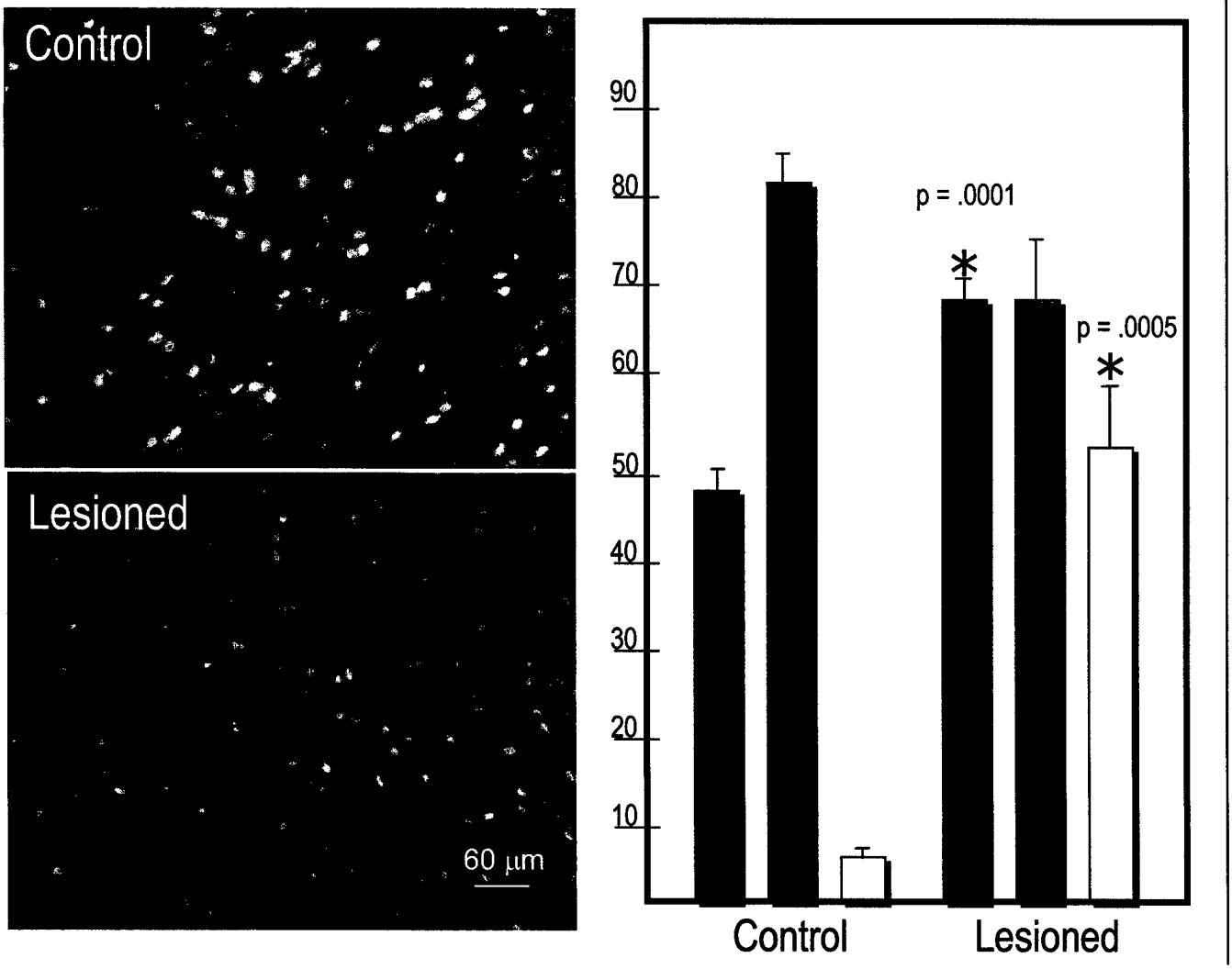

Striatal DOPAC Levels
Mean DOPAC (ng/mg protein)
18
16
14
12
10
8
6
4
2
0
p = .0002
*
p < .0001
*
Acute intact
Acute lesion
Chronic intact
Chronic lesion
TMT-Treated Experimental Groups


Striatal DOPAC/DA Ratio
Mean (ng/mg protein)
0.12
0.1
0.08
0.06
0.04
0.02
0
Acute intact
Acute lesion
Chronic intact
Chronic lesion
TMT-exposed Experimental Groups

Other Findings in the Preclinical PD Model

We examined other striatal responses 4 weeks following the infusion of 6-OHDA. We examined one of the common elements affected by apoptosis, caspase-3. Caspase-3 is a key enzyme that initiates programmed cell death. Activation of caspase-3 requires proteolytic processing of its inactive zymogen and thus cleaved caspase-3 detection is an index of pre-apoptotic activity in cells. We purchased cleaved caspase-3 antisera (Cell Signaling Technology). To assess whether the caspase-3 staining occurred in neurons, we purchased a monoclonal antibody against NeuN (a neuron-specific nuclear protein marker, Chemicon). These studies are **preliminary,** but we include them to demonstrate that significant changes occur in the striatum of the preclinical PD model.

Figure 10. Antisera against caspase-3 were detected using Cy3 (red) secondary antisera; NeuN is visible as Cy2 (green) staining. The images were merged electronically, off-line. A blinded observer counted the **red** caspase-3 cells, **green** NeuN cells, and **yellow** combined labeled cells in 12 different image pairs from a single 4-week 6-OHDA lesioned animal. Statistically significant changes were detected in neuronal cleaved caspase-3 expression (asterisks) in the lesioned versus the control striatum. These are visible as yellow cells, and are plotted according to their color, as the mean $\pm$ S.E. of cell numbers. Cleaved caspase-3 also is elevated in other striatal elements that are not neurons (red cells and red bar in the histogram).



Discussion:

The data showed that intrastriatal infusion of 6 μgm 6-OHDA at one site provided a mild insult to the DA nigrostriatal pathway, resulting in losses of DA at the cell body and terminal regions to produce a preclinical animal model at 1-week. The effect is more profound within the striatum because this is the site of the neurotoxin infusion, however both sites demonstrate substantial losses in the neurotransmitter and its turnover (measured by the ratio of [DOPAC] to [DA]). This argues that our unilateral neurotoxin paradigm will alter the DA nigrostriatal pathway functioning, even though animals do not demonstrate overt behavioral phenotypes. Our experimental approach has been designed to model the preclinical phase of PD followed by the progressive onset of symptoms in humans, who demonstrate anomalies in DA metabolism and function during the early stages of the disorder without explicit behavioral symptoms. The DA biochemical HPLC changes in the rats are supported by immunohistochemical detection of TH, the rate-limiting enzyme in catecholamine biosynthesis. Examination of postsynaptic striatal elements showed little difference in D1, D4, D5 receptor protein staining. However there is a consistent increase of D2 DA receptor protein staining and elevation in cyclic AMP levels within the neurotoxin treated striatum at 1 week. At the same time there is a decrease in staining intensity for the D3 DA receptor protein subtype. We have presented these data as photomicrographs and also as numerical values derived from luminosity histograms of the fluorescence intensity of the immunohistochemical experiments. However, the morphological data need to be corroborated in more animals to fully confirm the changes in levels of D2 and D3 DA receptors and cyclic AMP staining. We plan to initiate western analysis for this purpose. The data for the one-week time point was based upon analysis of 9 animals for HPLC biochemistry and 4 animals for immunohistochemical anatomy.

At the four-week time point, HPLC confirmed that 6 μgm of intrastriatal 6-OHDA still provided a 50% decrement in striatal DA levels. Thus we feel that this treatment produced a stable loss of the neurotransmitter in our preclinical PD model. Postsynaptic indices of the striatum demonstrate that the DA receptors on the 6-OHDA treated side recover to near equivalent levels to the control side with the notable exception of the D2 receptor, which remains slightly elevated on the lesioned side. Additionally, cyclic AMP stays elevated at the 4-week time point. These outcomes are based upon evaluation of 9 animals for HPLC biochemistry and 9 animals for immunohistochemical anatomy. We will also confirm these changes using westerns for the DA receptors as another method to validate our findings. These data suggest that the D2 receptor is the most sensitive to loss of DA, and this has some support in the literature using larger DA-depleting lesions (Gerfen et al, 1990; Minowa et al, 1994).

We also have initiated studies to determine other parameters of striatal functioning following subtotal loss of DA. These include examination of other PD related components, such as parkin- and cypin-containing elements. This survey has not provided any consistent information so far for these two proteins. Other preliminary data has shown that the neurotoxin-treated striatum is more poised to initiate programmed cell death, based upon examination of a keystone enzyme in the apoptosis cascade, caspase-3. The activated (e.g., cleaved) form of caspase-3 is elevated significantly in the lesioned striatum and a majority of the enhancement is selectively associated with neurons. We plan to continue this avenue of investigation, and have undertaken experiments to determine whether the enhancement of cleaved caspase-3 is due to the disruption of the striatum by the neurotoxin (we have sham-infused animals to evaluate), or actually due to DA deafferentation (we have infused 6-OHDA into the substantia nigra).

The use of a species relevant predator odor provides a novel method to produce stress in lab rodents. This behavior appears inborn, as all naïve animals exposed to minute quantities of the odorant (TMT) exhibited a vigorous behavioral response. The benefit of employing this compound as a secondary stressor is that no prior behavioral training or shaping of the animals is needed; thus enhancing the standardization of the secondary stressor event. The literature reports that TMT has selective effects on the DA pathways of the olfactory system and the hypothalamic-pituitary axis that indicate a healthy stress response occurs in rodents (Morrow et al, 2000a; ibid 2000b; Hayley et al, 2001). Our studies showed that TMT in the acute paradigm did not change DA levels or turnover in the partially lesioned nigrostriatal pathway. HPLC analysis of the chronic TMT design showed that DOPAC levels are not produced as abundantly. This outcome suggests that DA may possibly be degraded using an alternative biochemical pathway, or that the nigrostriatal system has recovered from the mild lesion by 9 weeks, and the TMT has no effect on DA turnover. We will repeat this experiment using a more aggressive TMT exposure regimen (twice per week), and validate HPLC with morphological analyses that have not been completed yet. The morphological studies have the advantage of detecting more subtle differences in the cellular distribution patterns of targeted striatal indices of nigrostriatal function. We will examine the apoptosis indicators in the TMT-treated animals, and remain highly optimistic that the findings will describe which elements of the system tolerate a secondary stressor following subtotal DA loss.

Summary of Key Accomplishments:

- Unilateral infusion of 6 μgm (2 μl of 3 mg/ml) of 6-OHDA produced a statistically significant loss of DA in the striatum; HPLC values showed 154 ng/mg protein DA on the intact side (n = 9) versus 56 ng/mg protein on the neurotoxin-treated side (n = 9, p <.0001). Lesser changes occurred in the substantia nigra, measured in these same animals. **TASK 1**
- Morphological assessment of the striatum 1 week following 6-OHDA treatments demonstrated nearly equivalent staining patterns and intensity for the D1, D4, and D5 DA receptor proteins. D3 DA receptor protein was decreased on the side of the neurotoxin infusion. The D2 DA receptor was elevated on the neurotoxin-treated side, as was the cyclic AMP second messenger. DA terminal losses were assessed using TH immunofluorescence and showed a substantial decrement in staining within the neurotoxin-treated striatum compared to the intact side. These data were obtained from 4 experimental animals. **TASK 2**
- Unilateral infusion of this same amount of neurotoxin (6 μgm) produced ~50% loss of DA 4 weeks after the neurotoxin treatment; HPLC values showed 200 ng/mg protein DA on the intact side (n = 8) versus 92.7 ng/mg protein on the neurotoxin-treated side (n = 8, p < .0001). The changes in the substantia nigra were not as dramatic in these same animals. **TASK 1**
- Morphological evaluation of the striatum 4 weeks after the 6-OHDA infusion demonstrated that the D2 DA receptor staining and expression were still elevated as was the cyclic AMP signal at this time point within the neurotoxin–treated striatum compared to the intact side. No other changes were detected using immunofluorescence for the DA receptor subtypes. **TASK 3**
- Treatments with the species-relevant predatory odor TMT showed remarkable behavioral avoidance by the exposed rats. Two experimental designs were used, an acute 24-hour survival, and a chronic, 5 week repetitive weekly exposure sequence. The HPLC analysis showed that the odorant decreased the breakdown of DA to DOPAC, but only in the chronic TMT treatment group. The values were not statistically significant. Morphological evaluation of this experimental design is under analysis. **TASK 4**

- The 50% DA depletion produces profound effects on the pre-apoptotic enzyme caspase-3 in the 4 week intrastriatal 6-OHDA animals. Our studies demonstrate a statistically significant 40% increase in the expression of cleaved caspase-3 in the DA-deafferented striatum, and this elevation is confined predominantly to striatal medium sized neurons, assessed through double labeling immunofluorescence using NeuN as a neuron-specific marker. Cell diameters on the lesioned side were slightly reduced, but not statistically so, indicating some atrophy due to intrastriatal infusion may occur.

Reportable Outcomes:

We will present a poster to the Chicago Chapter of the Society for Neuroscience (February 15, 2002) on the enhancement of apoptotic indicators in striatal neurons following partial intrastriatal 6-OHDA infusions. This work is a direct result of the DoD Neurotoxin Program funding.

A manuscript on the development of the D5 DA receptor null mutant mouse is under revision to *Nature Neuroscience*. Hollon TR, Bek MJ, Lachowicz JE, **Ariano MA**, Mezey E, Soares-da-Silva P, Liu ZF, Grinberg A, Drago J, Westphal H, Jose PA, and Sibley DR (2002) Mice lacking D_5 dopamine receptors have increased sympathetic tone and are hypertensive.

A manuscript on the inter-protein association of the D1 DA receptor and neurofilament M is submitted to the *Journal of Neuroscience*. Kim O-J, **Ariano MA**, Sibley DR, Neurofilament-M interacts with the D_1 dopamine receptor to regulate cell surface expression and desensitization.

Conclusions:

The reviewer felt our productivity was low in the 2nd year of the grant. We feel that the reviewer meant our *published* productivity has been minimal on the grant. However, we performed a substantial number of experiments in the second year of the grant, and the redesigned approach only began in late December 2000. We have obtained a huge amount of data that documents our PD animal model morphologically, and now feel that the model is characterized by HPLC and meets our criterion of 50% striatal DA loss at 4 weeks, consistently. We should now be able to generate reproducible data on the localization and expression of the DA receptor subtypes and apoptotic markers to produce a manuscript of significance and novelty to the field in the near future.

The reviewer questioned our use of TMT and whether our testable outcomes were now appropriate to evaluate the model. It is not clear what the reviewer's criticism is. We have not completed the examination of the TMT stressor event, and are still attempting to produce a reliable and rationale exposure sequence. Thus we cannot answer this concern presently. We will be in a better position to address this issue in the next reporting period.

We have just commenced using intranigral malonate infusions to establish a dose that will provide a 50% loss of striatal DA at the 4-week time point. Three different doses in 9 animals per group have been prepared. These animals will be sacrificed on 14 January 2002, and the striata and substantia nigras will be examined using HPLC. We will need to generate a group of animals for morphological evaluation once we have established the appropriate dose of intranigral malonate to employ.

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Appendix:

Letter requesting change in the SOW for the research program.

Email response approving the SOW by Dr. Karl Friedl, LTC US Army.

Finch University of Health Sciences / The Chicago Medical School

Marjorie A. Ariano, Ph.D.
Professor of Neuroscience

28 November 2000

Ms. Judy Pawlus
Office of the Deputy Chief of Staff
Information Management Branch
Department of the Army
US Army Medical Research and Materiel Command
504 Scott Street
Fort Detrick, MD 21702-5012

Dear Ms. Paulus

I am writing in reference to my award, # DAMD17-99-1-9542. It has become apparent to me that the original research plan in this award is hindering our progress and not allowing us to investigate fully the questions that we have proposed to address. After spending considerable time at the recent Society for Neuroscience meeting in New Orleans speaking with colleagues and experts in the field regarding specific concerns, I would like to propose a revision to the research plan. I believe the revision outlined below will assure results that are more consistent with current behavioral studies. The rationale for this change is noted below with justifications.

1. Unilateral lesions of the dopaminergic nigrostriatal pathway should be employed rather than a bilateral design. While Parkinson's disease in humans does develop initially as a unilateral disturbance and then progresses to bilateral involvement, the unilateral rodent lesion model has given useful information for the past 40 years and is the gold standard for animal studies of the movement disorder. The ability to produce a dopaminergic chemical imbalance in the nigrostriatal pathways by unilateral lesioning enables testing of specific dopaminergic behaviors in rodents that can be quantified readily.
2. Bilateral lesions have mandated special dietary and husbandry requirements that have delayed progress of our research. It also is evident that these special needs impede the use of two of the originally proposed time points (e.g., 24 hours and 3 days). Unilateral lesions are less challenging to the animals' post-surgical recovery. No special dietary considerations need to be implemented, either before the lesioning to introduce the diet to the animals, nor afterwards to maintain their body weights.
3. A unilateral lesion will provide an internal control in that the contralateral side can be used to assess changes in various morphological and chemical parameters, instead of using a naïve/control group of rats. This will decrease the number of animals necessary to perform the study.
4. While we have established the amount of striatal 6-hydroxydopamine that can be used to provide a bilateral 50% loss of striatal dopamine one week following the surgery, our progress has been slow due to a number of issues. Paramount among these deterrents is the inconsistency of the bilateral lesions within a single animal, regardless of the experience of the surgeon producing the neurotoxin depletion of the transmitter.

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Marjorie A. Ariano, Ph.D.
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It is absolutely imperative that we perfect the lesioning task before initiation of behavioral testing of the animals. If we cannot ensure reliable and consistent 50 % losses of striatal dopamine using the two different neurotoxin infusion paradigms (6-hydroxydopamine in the striatum and malonate in the substantia nigra), behavioral testing will not have a meaningful outcome. Thus the lesion paradigm is the first step of this project.

We are in the process of establishing collaboration with an expert in the field of dopamine behavioral assessment, Dr. Timothy Schallert (University of Michigan). He will teach us to quantify nigrostriatal dopaminergic losses using a very simple, straightforward behavior that assesses the number of paw touches by the animal in a vertical Plexiglas cylinder. Rats do not use the paw on the dopamine lesioned side compared to the intact dopaminergic nigrostriatal pathway, and Dr. Schallert has correlated this relationship with the chemical loss of the neurotransmitter levels in the striatum. This experimental design precludes the need to shape the animals' behavior on the task prior to the surgery. Moreover the natural tendency for the rodent to explore the cylinder will allow us to assess the rats' depletion without food deprivation to motivate the behavioral sequence. Thus we will be able to determine the extent of the dopaminergic depletion prior to sacrifice of the animal and not as a retrospective analysis. All of these amendments to our present experimental design will speed the progress of the research, improve the consistency of our data, and importantly, maintain a robust health in our lesioned animals.

We would like to initiate these changes quickly so that we will have an interesting and coherent report to share with our colleagues at the upcoming March 2001 meeting in Potomac, MD. Thank you for your cooperation and assistance in this matter.

Sincerely,

Marjorie A. Ariano, Ph.D
Professor of Neuroscience
Principal Investigator for the Project

Kathy Steece-Collier, Ph.D
Assistant Professor of Neuroscience
Co- Principal Investigator for the Project

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Marjorie Ariano

From: "Friedl, Karl E LTC USAMRMC" <Karl.Friedl@DET.AMEDD.ARMY.MIL>
To: "'Marjorie Ariano'" <Marjorie.Ariano@finchcms.edu>
Cc: "Yourick, Debra L Dr. WRAIR-Wash DC" <Debra.Yourick@NA.AMEDD.ARMY.MIL>; "Pawlus, Judy K Ms USAMRMC" <Judy.Pawlus@DET.AMEDD.ARMY.MIL>; "Miles, Cheryl R Ms USAMRAA" <Cheryl.Miles@DET.AMEDD.ARMY.MIL>; "Grate, Stephen J Dr SHERIKON" <Stephen.Grate@DET.AMEDD.ARMY.MIL>; "Burdette, Buffy J Ms SHERIKON" <Buffy.Burdette@DET.AMEDD.ARMY.MIL>
Sent: Thursday, April 05, 2001 4:43 PM
Subject: RE: "SOW" DAMD17-99-1-9542

If I understand your request correctly (28 Nov 00), you asked to switch to unilateral lesioning and noted a need to perfect your lesioning technique. You also asked to add Dr. Schallert from the University of Michigan as a consultant to help with your behavioral measurement techniques. There is no change in budget and no change in schedule. If this is the case, this email can certify that we will accept your experimental modifications without further delay; these are sensible adjustments.

Ms. Cheryl Miles in the contracting office may formalize this with a contract modification although I don't believe that will be necessary; Ms. Judy Pawlus is the correct point of contact for your periodic progress reports; and Dr. Debra Yourick at Walter Reed Army Institute of Research is your technical point of contact as the Contracting Officer's Representative.

On your previous annual report, Dr. Yourick noted that you had some experimental setbacks but felt that everything was properly executed and was scientifically acceptable. An outside review that we also conduct on each annual report was a bit harsher in their assessment of progress (you should have received a copy of that review) but you have assured me that you are making good progress and will be on schedule to meet all of your research objectives. Please feel free to discuss technical issues and concerns with Dr. Yourick (copied on this email) as you progress. We are interested in helping you to succeed and it sounds like you are on the right track.

Thank you again for taking time from your schedule to participate in the workshop. We have received enough favorable comments that we will definitely consider a reprise if the program continues to grow.

Karl Friedl

-----Original Message-----
From: Marjorie Ariano [mailto:Marjorie.Ariano@finchcms.edu]
Sent: Thursday, April 05, 2001 2:47 PM
To: karl.friedl@det.amedd.army.mil
Cc: stephen.grate@det.amedd.army.mil
Subject: "SOW"

LTC Friedl,

I still have not received anything in writing regarding the approval of my amended SOW. I presume it is "in the mail?"

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